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Study Title

R54 (3rd edn) Clofentezine:
Analytical Method for the Determination of Free
Clofentezine Residues in Milk & Animal Fat by High Performance
Liquid Chromatography

Data RequirementsGuideline: 171-4Authors

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5/6/89

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RESID/89/50

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C).

Company: NOR-AM Chemical Company
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Title: Registration Project Leader

Date: 5/4/89

Christopher Davis
Signature

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REPORT AUTHENTICATION

I, the undersigned, hereby declare that the work to which this report refers was performed according to the procedures herein described and to the prevailing standard operating procedures, and this report provides a correct and faithful record of the results obtained.

..... *P. S. Snowden*
Study Director

..... *6th May 1989*
Date

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NOTES

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Originating department RESIDUE ANALYSIS
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Security category	Registration reference NC 21 314/R54 (3RD EDITION)
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Study No. 073/01/001	Report No. RESID/89/50
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Report title ANALYTICAL METHOD FOR THE DETERMINATION OF FREE CLOFENTEZINE RESIDUES IN MILK AND ANIMAL FAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ANALYTICAL METHOD FOR THE DETERMINATION OF FREE CLOFENTEZINE RESIDUES
IN MILK AND ANIMAL FAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ANALYTICAL METHOD FOR THE DETERMINATION OF FREE CLOFENTEZINE RESIDUES
IN MILK AND ANIMAL FAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

1 SUMMARY OF PERFORMANCE CHARACTERISTICS

1.1 Compound determined

Clofentezine only.

1.2 Type of sample

Whole milk peritoneal and subcutaneous fat from dairy cows.

1.3 Basis of method

Solvent extraction of clofentezine from substrate. Clean-up by acetonitrile/hexane partition followed by elution through a Sep-pak silica cartridge. Final determination by normal phase high-performance liquid chromatography (HPLC) with UV absorption detection.

Residues may be confirmed by re-determination under reverse phase conditions.

1.4 Calibration

Linear over the tested range of 0.5 to 10.0 µg/ml concentrations of clofentezine in standard solutions (5 to 100 ng clofentezine injected into the chromatograph).

1.5 Recovery efficiency

Mean recovery efficiencies of 88.1% and 79.8% were obtained from recovery tests with untreated samples of milk and fat, respectively, at fortification levels between 0.01 and 0.2 mg/kg clofentezine.

1.6 Precision

The standard deviations for these data were 6.6% (milk) and 9.4% (fat).

1.7 Limits of determination

Estimated as 0.01 mg/kg for clofentezine in both milk and fat.

1.8 Limit of detection

Estimated as 1.5 ng injected into the HPLC, corresponding to a residue of 0.002 mg/kg clofentezine (assuming a 50 g sample aliquot) or 0.004 mg/kg (assuming a 25 g sample aliquot).

1.9 Time for analysis

Analysis of a batch of six samples takes approximately one day from weighing out the samples to preparation of the final solution for HPLC determination, which itself takes a further 1½ hours.

2 INTRODUCTION

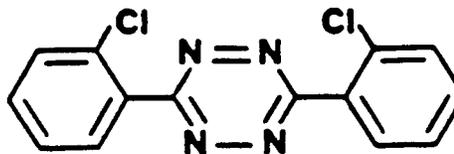
The acaricide clofentezine is being developed for use primarily on top fruit. Processing procedures for this fruit commonly involve production of dried apple pomace for incorporation into animal feeds. It is therefore of importance to determine any residue levels occurring in both animal tissues and milk.

The particular analytical method described in this report has been developed specifically for the purpose of monitoring active ingredient residues only in samples of milk and animal fat. Determination of clofentezine animal metabolite residues in these substrates is the subject of a separate report (1), as is the determination of clofentezine and metabolite residues in animal tissues (2).

3 PHYSICAL AND CHEMICAL DATA

3.1 Clofentezine

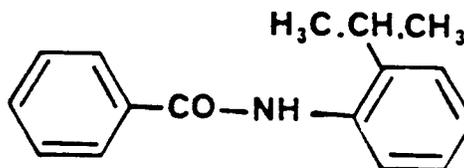
Chemical structure :



Systematic name (CA, IUPAC)	:	3,6-bis-(2-chlorophenyl)- 1,2,4,5-tetrazine
Molecular formula	:	$C_{14}H_8N_4Cl_2$
Relative molecular mass	:	303.0
Solubilities (25°C)	:	
Water		<1 mg/l
✓ Acetone		5 g/l
✓ Chloroform		50 g/l
Benzene		2.5 g/l
Ethanol		1 g/l
Hexane		1 g/l
Vapour pressure	:	13 fPa at 25°C (approx. 10^{-16} mm Hg)

3.2 NPPBA (HPLC marker compound; normal phase conditions)

Chemical structure :



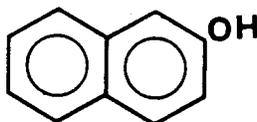
Systematic name : N-2-(2-propyl)phenylbenzamide
(CA, IUPAC)

Molecular formula : $C_{16}H_{17}NO$

Relative molecular mass : 239.0

3.3 2-Naphthol (HPLC marker compound; reverse phase conditions)

Chemical structure :
(IUPAC)



Systematic name : 2-naphthol

Molecular formula : $C_{10}H_8O$

Relative molecular mass : 144.0

4 PRINCIPLE OF METHOD

Analysis is restricted to the active ingredient only, clofentezine.

Minced fat samples are homogenised with a mixture of dichloromethane and methanol to extract clofentezine. Extraction of milk is based on the Mojonnier technique (3) for fat extraction and involves shaking with a mixture of hexane and diethyl ether after first breaking the milk fat globule membrane with potassium oxalate/ethanol.

Concentrated extracts of all sample types are then cleaned-up by acetonitrile/hexane partition to remove fat, followed by elution through a Sep-pak silica cartridge. Final determination is performed by normal phase HPLC, with detection of clofentezine by monitoring UV absorption at 268 nm. These conditions produce a suitably clean chromatogram, sufficiently free of any co-extractive interference.

Residues may be confirmed by re-determination under reverse phase conditions.

5 ANALYTICAL PROCEDURE

5.1 Reagents

5.1.1 Analytical reagents - see Appendix I

~~Metasil A filter aid~~

~~Sodium chloride, potassium oxalate - Analytical Reagent (A.R.) grade~~

Anhydrous sodium sulphate - Specified Laboratory Reagent (SLR) grade

5.1.2 Organic solvents

Diethyl ether, hexane, dichloromethane, propan-2-ol, methanol, ethanol. All 'Pesticide Grade' - see Appendix I.

Ethyl acetate, hexane, propan-2-ol, acetonitrile. All 'HPLC' Grade - see Appendix I.

5.2 Apparatus

The following items are used in this laboratory. Alternatives may be acceptable :-

Crypto Peerless food mincer.

MSE overhead drive homogeniser with 100 ml vortex beaker.

Buchi rotary evaporator.

Kuderna-Danish (K-D) evaporator with water bath.

MSE GF-8 centrifuge (with adapter to hold 380 ml screw-top PTFE containers).

Dri-block for solvent evaporation (under nitrogen stream).

Stuart flask shaker.

Vortex mixer.

Silica Sep-pak cartridges (Waters Associates Ltd - see Appendix I).

High Performance Liquid Chromatograph (HPLC) - see section 5.5.

5.3 Sample preparation

5.3.1 Fat

Samples should be minced through a Crypto-Peerless mincer whilst still frozen, thoroughly mixed and returned to deep freeze (-20°C) before further handling.

5.3.2 Milk

Samples should be stored frozen (-20°C) and thawed at room temperature prior to analysis.

5.4 Laboratory steps

5.4.1 Fortification of recovery samples

5.4.1.1 Prepare a stock solution containing 1000 µg/ml clofentezine in acetone. Store this solution under refrigeration (1°C) when not in use.

5.4.1.2 Use this stock solution to prepare working solutions of 1 and 10 µg/ml of clofentezine in acetone.

5.4.1.3 Fortify recovery samples at the specified stage (section 5.4.2 or 5.4.3) according to sample type, by pipetting from a 1 or 10 µg/ml working solution of clofentezine onto the sample.

5.4.2 Extraction of clofentezine (fat)

5.4.2.1 Weigh 25 g of minced sample into a macerating flask and add anhydrous sodium sulphate (5 g). (Recovery samples should be fortified at this stage, using a solution of 1 or 10 µg/ml clofentezine in acetone.)

5.4.2.2 Add 1 + 9 v/v methanol + dichloromethane (70 ml) and macerate for 5 minutes. Filter under suction on a Buchner funnel through a pad of Metasil 'A', rinsing flask and filter cake with fresh solvent mixture (20 ml).

5.4.2.3 Repeat step 5.4.2.2 twice, combining filtrates in a 500 ml separating funnel.

5.4.2.4 Shake with water (100 ml) and saturated sodium chloride solution (10 ml). Allow to separate and run off lower dichloromethane layer into a conical flask.

5.4.2.5 Add fresh dichloromethane (80 ml) to aqueous phase, shake and allow to separate.

5.4.2.6 Dry combined dichloromethane extracts over anhydrous sodium sulphate (40 g) and filter into a Kuderna-Danish (K-D) evaporator through a fluted No. 4 filter paper. Rinse flask and filter with fresh dichloromethane (25 ml).

5.4.2.7 Reduce contents of K-D on a boiling water bath to less than 5 ml as far as possible, to remaining oil.

5.4.2.8 Proceed to section 5.4.4.

5.4.3 Extraction of clofentezine (milk)

- 5.4.3.1 Weigh 50 g of the thawed whole milk sample into a 380 ml PTFE screw-top centrifuge bottle. (Recovery samples should be fortified at this stage.)
- 5.4.3.2 Add 5% w/v potassium oxalate solution (10 ml), propan-2-ol (60 ml) and water (20 ml). Replace cap on bottle and shake for one minute. (May be done automatically using a flask shaker.)
- 5.4.3.3 Add 1:1 v/v diethyl ether : hexane (100 ml), replace cap and shake for 1 min.
- 5.4.3.4 Centrifuge at 2000 rpm for 5 mins.
- 5.4.3.5 Decant carefully into a 500 ml separating funnel. Run lower aqueous layer back in to centrifuge bottle. Collect organic layer in a conical flask. (Small quantities of ethanol may be added to effect a separation in the event of emulsions.)
- 5.4.3.6 Add 1:1 v/v diethyl ether : hexane (50 ml) and propan-2-ol (10 ml) to bottle, replace cap and shake for 1 min. Centrifuge at 2000 rpm for 5 mins.
- 5.4.3.7 Repeat steps 5.4.3.5 and 5.4.3.6.
- 5.4.3.8 Transfer combined organic extracts back to separating funnel. Wash twice with water (100 ml, 50 ml) and saturated sodium chloride solution (10 ml).
- 5.4.3.9 Dry organic extract over anhydrous sodium sulphate (40 g) and filter into a 500 ml round-bottomed flask through a fluted No. 4 filter paper. Rinse flask and filter with fresh hexane (25 ml).
- 5.4.3.10 Reduce contents of flask on a rotary evaporator at 50°C, until an oily residue remains.
- 5.4.3.11 Proceed to section 5.4.4.

5.4.4 Acetonitrile/hexane partition

- 5.4.4.1 Rinse extracts from section 5.4.2.8 or 5.4.3.11 into a separating funnel using hexane (100 ml for fat, 30 ml for milk).
- 5.4.4.2 Shake with acetonitrile (50 ml for fat, 25 ml for milk). Allow to separate and collect acetonitrile in a conical flask.
- 5.4.4.3 Repeat step 5.4.4.2 and discard hexane. Wash combined acetonitrile with fresh hexane (20 ml), subsequently discarded.
- 5.4.4.4 To acetonitrile add water (~~250 ml for fat,~~ 100 ml for milk). Shake three times with fresh hexane (70, 50 and 50 ml for fat; 50, 50 and 50 ml for milk).
- 5.4.4.5 Discard aqueous phase and run combined hexane extracts into a K-D evaporator through a fluted No. 4 filter paper containing anhydrous sodium sulphate (10 g). Rinse filter with fresh hexane (25 ml).
- 5.4.4.6 Reduce contents of K-D to less than 5 ml on a boiling water bath.
- 5.4.4.7 Further reduce extract to a volume of 1 to 2 ml on a Dri-block at 45°C under a stream of nitrogen.

5.4.5 Sep-pak clean-up

- 5.4.5.1 Transfer concentrated hexane extract onto a Sep-pak silica cartridge.
- 5.4.5.2 Rinse tube with hexane (2 x 1 ml) and transfer each in turn to the cartridge.
- 5.4.5.3 Elute with 1:4 v/v dichloromethane : hexane (10 ml) and discard this fraction.
- 5.4.5.4 Elute with 3:2 v/v dichloromethane : hexane (10 ml) and collect this fraction.
- 5.4.5.5 Take cleaned extract just to dryness using a Dri-block at 50°C and a stream of nitrogen.

5.4.5.6 Dissolve residue in appropriate volume (usually 0.5 or 1.0 ml) of marker solution (40 µg/ml of NPPBA in 9:1 v/v hexane : ethyl acetate - see section 5.6.1).

5.4.5.7 For automatic HPLC injection, transfer each solution to an autosampler vial.

5.5 Determination

The following operating conditions have been used successfully during development of this analytical method. However, these are not rigid requirements of the method and may be altered as necessary depending on specific applications or changes in resources.

Column : Lichrosorb Si-60-5; length 25 cm; i.d 4.6 mm

Guard column : Uptight C-130B (Upchurch Scientific Inc.)
packed with Whatman HC Pellosil (30-38 µm)

Pump : Varian LC 5010

Injector : Waters WISP 710A for automatic injection.
Varian manual loop injector for manual injection.

Mobile phase : Ethyl acetate (100 ml) diluted to 1 litre
with hexane; 2 ml iso-propyl alcohol added.

Flow rate : 2.0 ml/min.

Detector : Pye Unicam PU 4020 UV detector, set at
268 nm.
Output signal attenuated by integrator.

Data handling : Beckman CALS laboratory information
management system using PeakPro software
(Appendix II). Earlier methodology work used
a Hewlett-Packard 3388A reporting integrator
system for measurement of peaks and
calculation of results. Output from both
systems has been used to provide typical
chromatogram examples in Appendix III.

Injection : 15 µl
volume

Retentions : Clofentezine 3.9 to 4.5 mins
(approximate) NPPBA 6.3 to 7.6 mins

5.6 Confirmation of residues

5.6.1 Clofentezine residues may be confirmed by re-determination by HPLC using reverse phase conditions. Samples should be analysed exactly according to the procedures described in section 5.4 with the following modification at step 5.4.5.6 :

Dissolve final residue in HPLC marker solution for reverse phase operation (1.0 ml pipetted from a 10 µg/ml solution of 2-naphthol in acetonitrile - see section 5.7).

5.6.2 Determination of residues may then proceed using the reverse phase HPLC conditions outlined below.

Instrument	:	Varian LC 5010
Column	:	Spherisorb 5 ODS, 25 cm x 4.6 mm i.d.
Guard column	:	Uptight C-130B (Upchurch Scientific Inc.) packed with Whatman Pellicular ODS (37-53 µm)
Mobile phase	:	6:4 v/v acetonitrile:water + 0.1% acetic acid
Flow rate	:	1.5 ml/min
Detector	:	Pye Unicam PU 4020 U.V. detector, set at 268 nm
Injection volume	:	30 µl
Retention times	:	Clofentezine approx. 7.7 min 2-naphthol approx. 2.8 min

5.7 Calibration

- 5.7.1 Prepare stock solutions containing 1000 µg/ml of NPPBA (normal phase HPLC marker compound) in ethyl acetate and 1000 µg/ml of 2-naphthol (reverse phase HPLC marker compound) in methanol. Store under refrigeration (1°C) when not in use. From these stock solutions prepare working solutions of 40 µg/ml NPPBA in 9:1 v/v hexane : ethyl acetate, and 10 µg/ml 2-naphthol in acetonitrile respectively.

An HPLC marker compound is used purely to accommodate fluctuations in HPLC injection volume, and is recommended under circumstances where variations are more probable. However, the analytical method described in this report may be used without a marker compound, particularly if a fixed volume injection loop (20 µl) is incorporated into the HPLC system. Under these circumstances, each HPLC sample should be injected several times to demonstrate adequate reproducibility. Additionally, it is important that the final volume of the extract (section 5.4.5.6) is accurately made up to a known value and that evaporation losses are kept to a minimum from this point onwards.

- 5.7.2 Past experience of using a similar determination technique (4) with calibration standards containing from 0.5 to 10.0 mg/ml clofentezine has confirmed the excellent linearity of the detector U.V. absorption response over this concentration range, fitting an equation of the form $y = mx$ (where y = peak height ratio - see section 5.6.3, x = concentration, m = gradient). Therefore, only one point on this calibration need be determined in order to define the line (by calculation of m).
- 5.7.3 To define this line with sufficient accuracy, prepare at least three standards, each containing 10 µg/ml clofentezine, by pipetting 1 ml aliquots from a working solution of 10 µg/ml in acetone into separate sample vials.

5.7.4 Take each standard just to dryness using a Dri-block at 45°C and a stream of nitrogen. Dissolve the residue in 1.0 ml of marker solution (40 µg/ml NPPBA in 9:1 v/v hexane : ethyl acetate for normal phase HPLC, or 1.0 ml of 10 µg/ml 2-naphthol in acetonitrile for reverse phase confirmatory analysis) and inject into HPLC either manually or automatically.

5.8 Calculations

5.8.1 In each chromatogram measure the peak area of clofentezine and express this as a percentage of the marker peak area.

5.8.2 From a calibration line of this peak area ratio versus concentration, plotted from the standards, the concentration of clofentezine (c µg/ml) in the injected sample solution may be found.

5.8.3 Knowing the volume of this HPLC solution (V ml) and the weight of sample (W g) analysed, the residue (R) is defined as:-

$$R = \frac{C.V}{W} \text{ mg/kg (ppm, w/w)}$$

5.8.4 To determine recovery efficiency:

$$\text{Recovery} = \frac{C.V}{\mu\text{g clofentezine added}} \times 100\%$$

6 PERFORMANCE

6.1 Recovery efficiency

Individual recovery efficiency results for clofentezine in each sample type are listed in Table 1. These have been corrected for any apparent residues found in the respective control samples used for fortification.

6.2 Limit of determination

A level of 0.01 mg/kg clofentezine is the lowest concentration at which residues have been satisfactorily quantified in milk and fat in terms of recovery efficiency.

Analysis of control (untreated) samples (Table 2) has indicated that any apparent residues found are usually below this value which is therefore considered to be an appropriate limit of determination.

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Table 1

Recovery efficiency of clofentezine from fortified samples

Fortification level (mg/kg)	Recovery efficiency (%)	
	Milk	Fat
0.01	99, 89, 86, 95	92
0.02	74, 84, 86	64, 91, 81, 84
0.04	89	71, 86, 84
0.08	87, 85	
0.10	90	71
0.20	89	74
Mean	88.1	79.8
Std. dev.	6.6	9.4

Table 2

Sample type	Apparent residue level (mg/kg)
Milk	ND, ND, 0.001
Fat	0.005, 0.003, 0.02

ND denotes a non-detectable residue

6.3 Limit of detection

The smallest amount of clofentezine which is detectable by the liquid chromatograph using the operating conditions outlined in section 5.5 is estimated to be 1.5 ng. This limit of detection corresponds to an apparent residue level of 0.002 mg/kg for a 50 g sample weight and 0.004 mg/kg for a 25 g sample weight.

6.4 Typical chromatograms

Examples of typical chromatograms are included in Appendix III.

6.5 Confirmatory analysis

Several recovery efficiency tests have been performed using the confirmatory analysis HPLC conditions outlined in section 5.6. The results obtained are listed in Table 3, with typical chromatograms selected in Appendix III.

The elution of clofentezine by the reverse phase mechanism does not compromise chromatographic resolution or method performance, and provides a suitable means of confirming the presence of a residue.

Table 3

Recovery efficiency of clofentezine under confirmatory analysis conditions (reverse phase HPLC)

Fortification level (mg/kg)	Recovery efficiency (%)	
	Milk	Fat
0.04	76, 72	
0.08	74, 76	63, 84, 66
Mean	74.5	71.0

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6.6 Archives

All raw data relating to this study and a copy of this report will be filed under study number 073/01/007 in the archives of the Environment, Product Safety and Registration Group at Chesterford Park Research Station.

7 REFERENCES

- 1 SNOWDON, P.J., PEATMAN, M.H., Schering Report RESID/89/49 (May, 1989)
"ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF 4-HYDROXYCLOFENTEZINE IN MILK AND ANIMAL FAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY"
Registration reference Clofentezine/R182 (2nd Edition)
2. SNOWDON, P.J., Schering Report RESID/89/51 (May 1989)
"ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF CLOFENTEZINE AND 4-HYDROXYCLOFENTEZINE IN ANIMAL TISSUES BY GAS CHROMATOGRAPHY"
Registration reference NC 21 314/R72 (3rd Edition)
- 3 "ANALYTICAL METHODS FOR PESTICIDE, PLANT GROWTH REGULATORS AND FOOD ADDITIVES", Vol. 1 (1963), Ed. G. Zweig. pp. 557-559.
4. SNOWDON, P.J. and CROFTS, M., FBC Report RESID/81/33 (May 1981)
"ANALYTICAL METHOD FOR RESIDUES OF NC 21 314 IN APPLES"
Registration reference NC 21 314/R6 = C8

APPENDIX I

Reagent specifications

All obtained from Fisons Scientific Apparatus (FSA), Loughborough, Leics., unless otherwise stated. All specifications are taken from the appropriate catalogues.

I.1 Analytical reagents

Sodium chloride : (AR grade)	Assay 99.9% min Bromide <0.005% Nitrate <5 ppm Sulphate (SO ₄) <0.002%
Potassium oxalate : (AR grade)	Assay 99.5% min Sulphate (SO ₄) <0.01% Chloride (Cl) <0.001%
Sodium sulphate : (SLR grade)	Assay (after drying) 99% min Chloride (Cl) <0.01% Moisture <2.0%
Metasil 'A' filter aid :	Pure diatomaceous silica SiO ₂ 89.6% Al ₂ O ₃ 4.0% Fe ₂ O ₃ 1.5% Na ₂ O + K ₂ O 3.3% Other oxides <0.6%

I.2 Organic solvents

Pesticide grade :	No impurities causing interfering peaks greater than that given by 10 ng/l aldrin (by ECD) or 100 ng/l parathion (by TED); max. residue <2 ppm, total phosphorus (by GC) <0.0005 ppm; H ₂ O content <0.05%.
HPLC grade hexane :	95% n-hexane Acidity (acetic) <0.001% Residue after evaporation <2 ppm Water <0.01% Max. absorbance :- 200 nm 0.30 A.U. 210 nm 0.10 A.U. 220 nm 0.025 A.U. 230 nm 0.01 A.U. 240 nm 0.005 A.U. 250 nm 0.005 A.U.

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HPLC grade ethyl acetate :
Assay 99.5% min
Acidity (acetic) <0.01%
Residue after evaporation <2 ppm
Water <0.02%
U.V. cut-off (1.0 A.U.) 255 nm
Max absorbance :-
260 nm 0.10 A.U.
270 nm 0.025 A.U.
280 nm 0.005 A.U.

HPLC grade propan-2-ol :
Acidity (acetic) <5 ppm
Residue after evaporation <2 ppm
Water <0.05%
U.V. cut-off (1.0 A.U.) 205 nm
Max absorbance :-
250 nm 0.01 A.U.
260 nm 0.005 A.U.
270 nm 0.005 A.U.

HPLC grade methanol :
Assay 99.8% min
Acidity (acetic) <0.001%
Residue after evaporation <5 ppm
Water <0.05%
Max absorbance :-
210 nm 0.40 A.U.
220 nm 0.20 A.U.
230 nm 0.10 A.U.
240 nm 0.15 A.U.
250 nm 0.005 A.U.
260 nm 0.005 A.U.

I.3 Sep-pak silica cartridges

Silica solid phase extraction columns : Supplied by Waters Associates,
Part No. 51900.

APPENDIX II

Data handling

All chromatographic data produced during this study were handled by a 'PeakPro' chromatography software package operating within a Beckman CALS laboratory information management system running on Hewlett-Packard computer hardware (A-900 series HP1000 processor unit).

Each chromatogram is collected into a data file as a series of data points after conversion of the chromatograph's analogue signal into digital format. This is accomplished by conversion interfaces known as 'Digimetries', supplied by Beckman LAO Ltd. The rate of data collection (points per second) and the collection interval (minutes) are set by the user and stored in a separate acquisition method file named according to the convention AC ----, where '-' represents a digit. The digitised chromatogram obtained (raw data file) is named according to the convention **--R, where * represents any letter; i.e. acceptable file names range from AA001R to ZZ999R.

Interpretation of the raw data file in terms of peak areas/heights is carried out by application of an integration algorithm from within PeakPro.

Individual parameters set for correct interpretation, together with appropriate annotation (peak names, etc.) are stored in GC method files named according to the convention GC---. The GC method file may include calibration data necessary to express final results in the required terms.

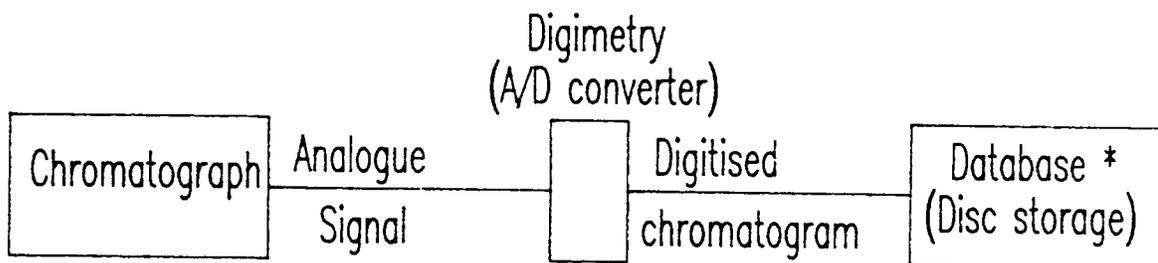
Once a raw data file (e.g. AA001R) has been analysed by a specific GC method (e.g. GC0001), the results are stored in a corresponding file differentiated only by the suffix 'C' (i.e. AA001C for the above example. The 'R' and 'C' files therefore remained linked by the common characters AA001). The results file ('C' file) also holds a snapshot of the GC method as used to perform the analysis of the raw data. If the GC method is altered and re-analysis is invoked, a snapshot of the revised parameters are stored in the 'C' file. The route to the results is therefore always retained.

Once acquired, chromatographic information may be re-displayed and reported ad-infinitum. For example, expanded plots may be produced at any time in order to 'zoom-in' on areas of interest.

A schematic summary of the above description is shown in Figure 1.

Figure 1

Schematic summary of data handling



* Files :

e.g. AA001R - raw data file

AA001C - corresponding results file

AC1234 data acquisition method

GC1234 GC method (baseline fit, component names, calibration)

APPENDIX III

Typical chromatograms

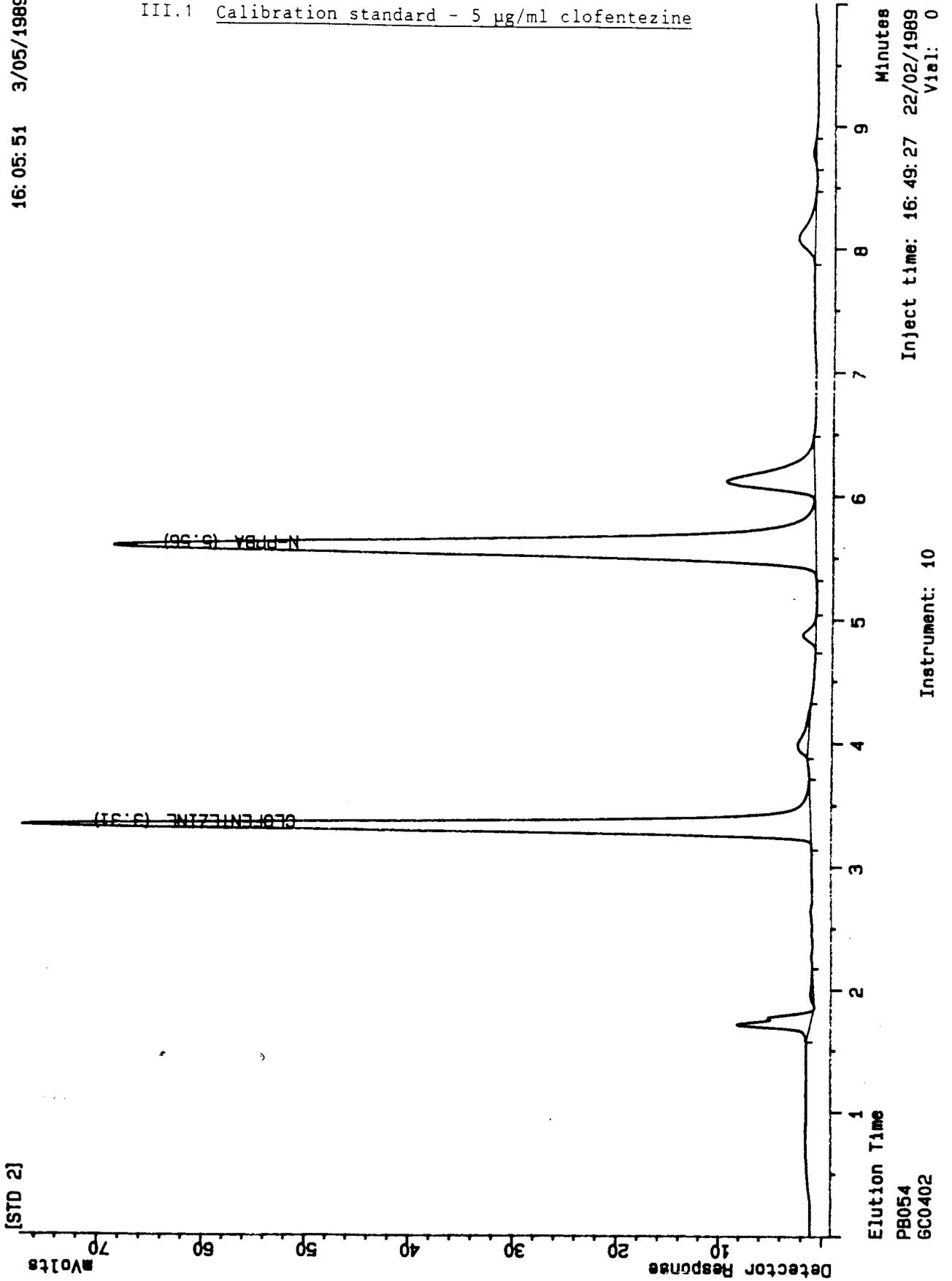
Examples of typical chromatograms obtained with this analytical method have been included in the following order.

Chromatogram	Type	Analysis reference	Description
III.1	Calibration std.	30BRHP	5 ug/ml clofentezine
III.2	Calibration std., confirmatory HPLC	41BRGP	5 ug/ml clofentezine
III.3	Control milk extract	30BRAP	Apparent residue 0.001 mg/kg
III.4	Milk recovery test	30BEBP	89% recovery efficiency at 0.08 mg/kg
III.5	Control milk extract, confirmatory HPLC	50BRAP	Non-detectable residue
III.6	Milk recovery test, confirmatory HPLC	50BRBP	76% recovery efficiency at 0.04 mg/kg
III.7	Control fat extract	13AOAP	Apparent residue 0.003 mg/kg
III.8	Fat recovery test	55AOBP	91% recovery efficiency at 0.02 mg/kg
III.9	Control fat extract, confirmatory HPLC	51BRAP	Non-detectable residue
III.10	Fat recovery test, confirmatory HPLC	51BRFP	66% recovery efficiency at 0.08 mg/kg

In those chromatograms which have not been annotated with peak names, peak A represents clofentezine (NC 21314) and peak B represents the normal phase HPLC marker compound NPPBA (C₁₆H₁₇NO).

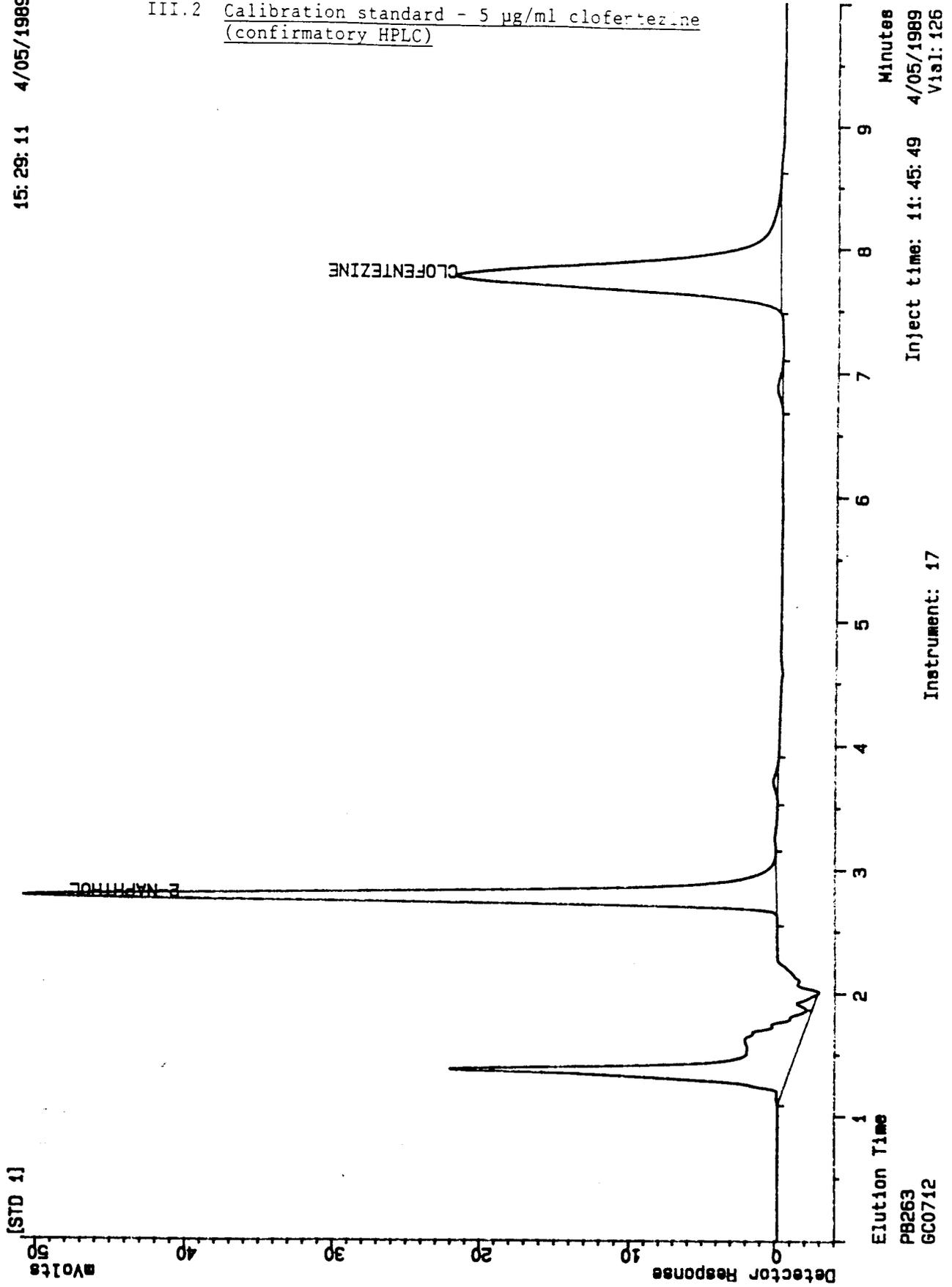
16: 05: 51 3/05/1989

III.1 Calibration standard - 5 µg/ml clofentezine



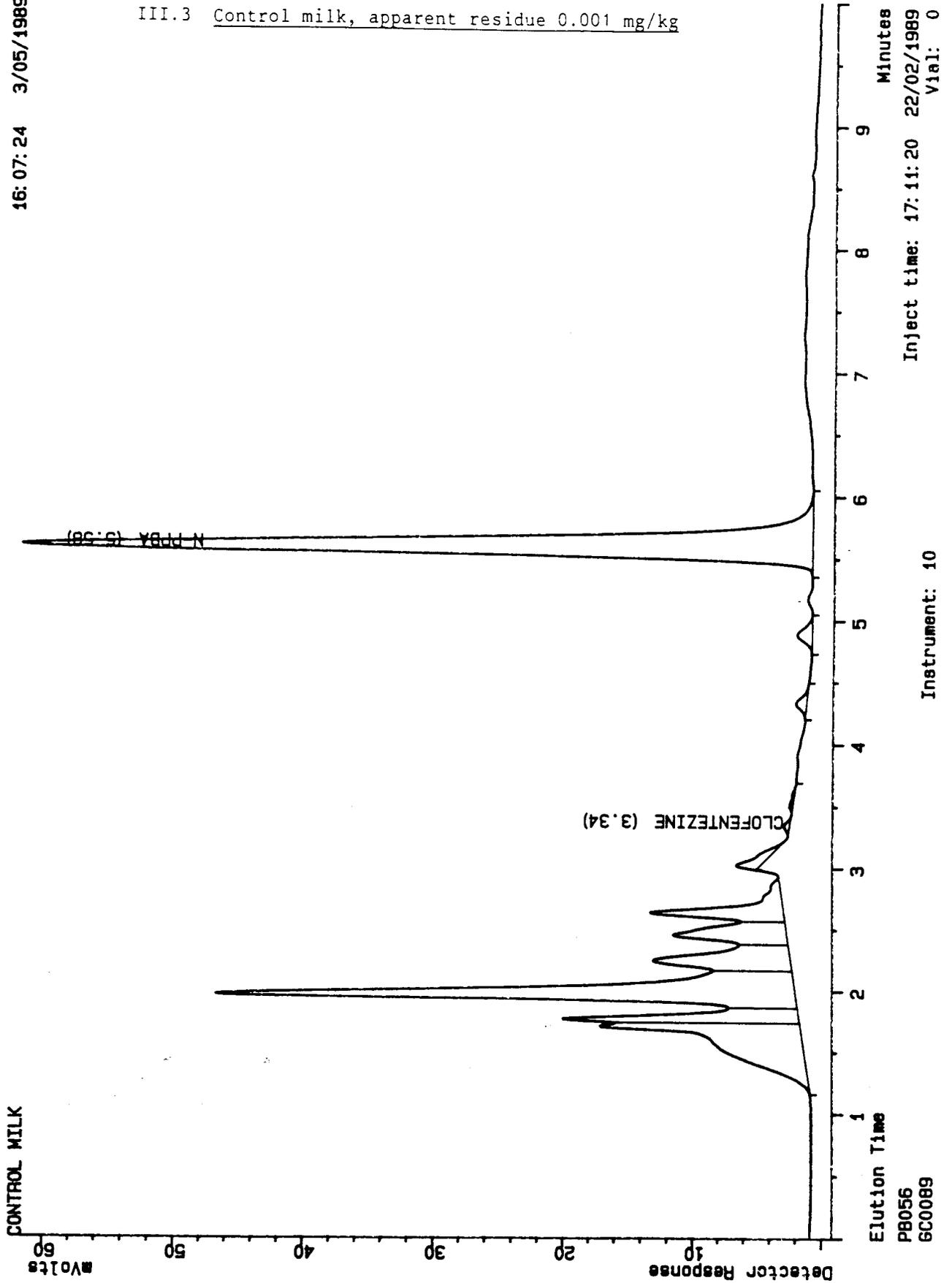
15: 29: 11 4/05/1989

III.2 Calibration standard - 5 µg/ml clofentezine
(confirmatory HPLC)



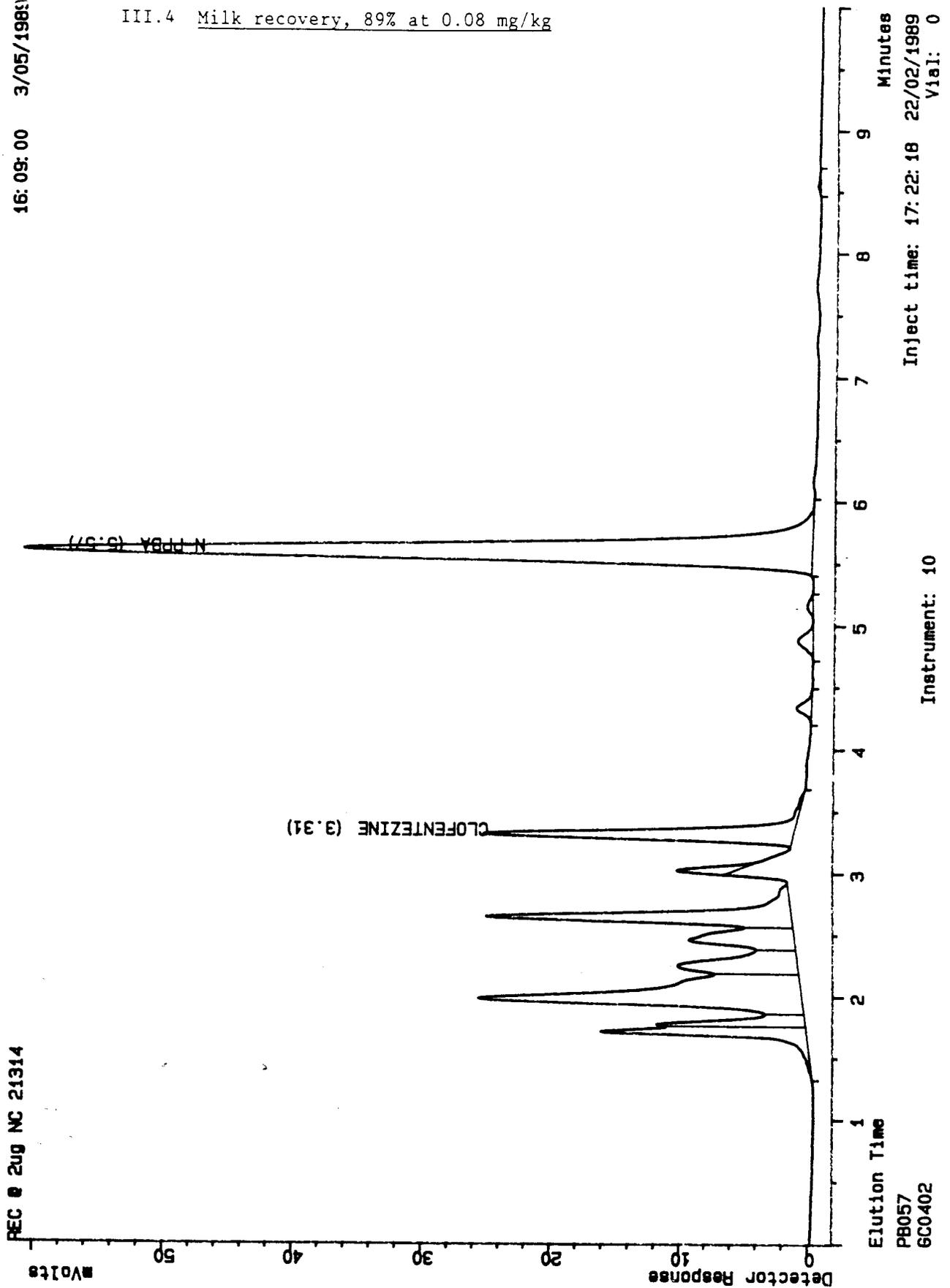
16: 07: 24 3/05/1989

III.3 Control milk, apparent residue 0.001 mg/kg



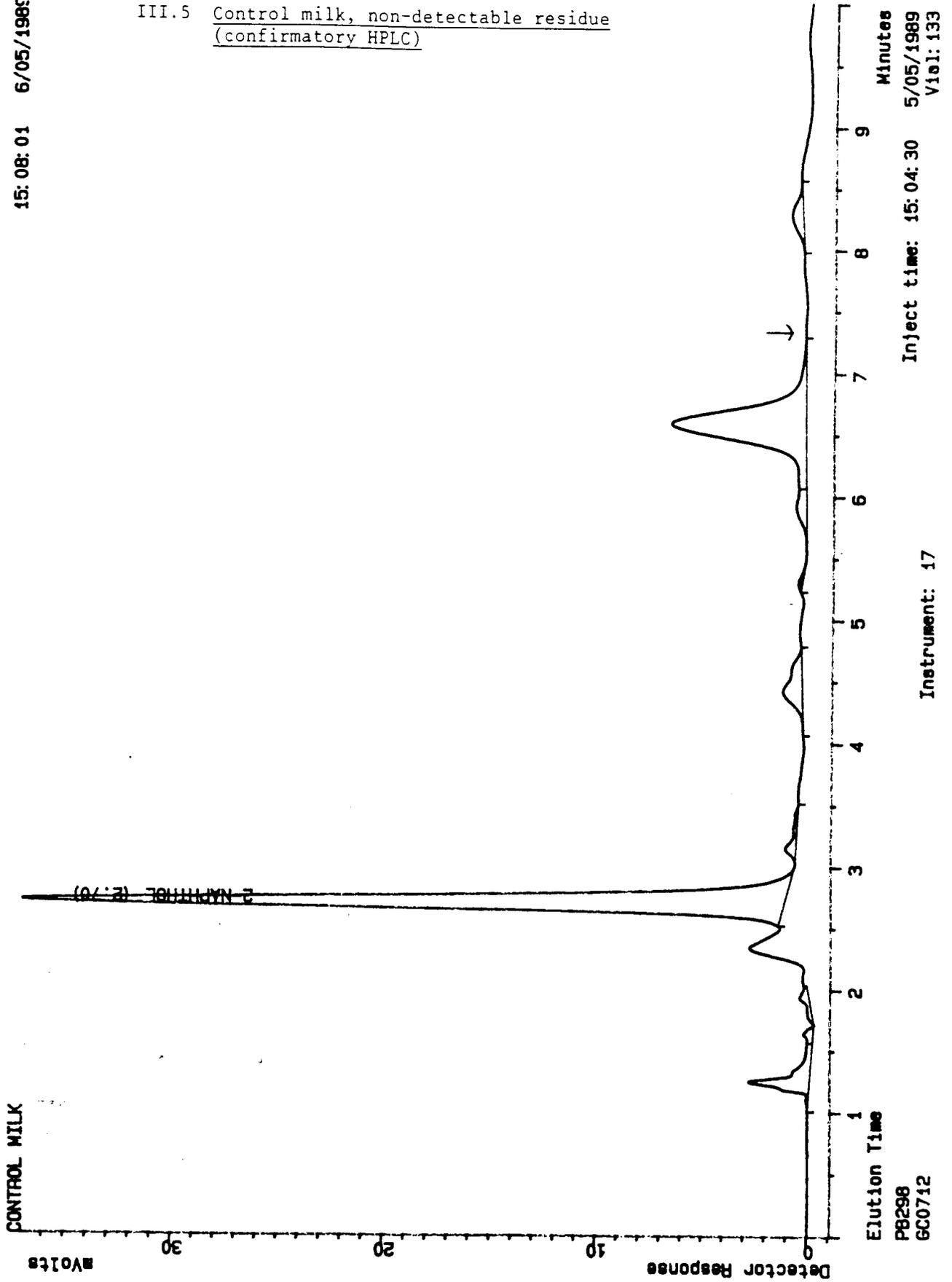
16: 09: 00 3/05/1989

III.4 Milk recovery, 89% at 0.08 mg/kg



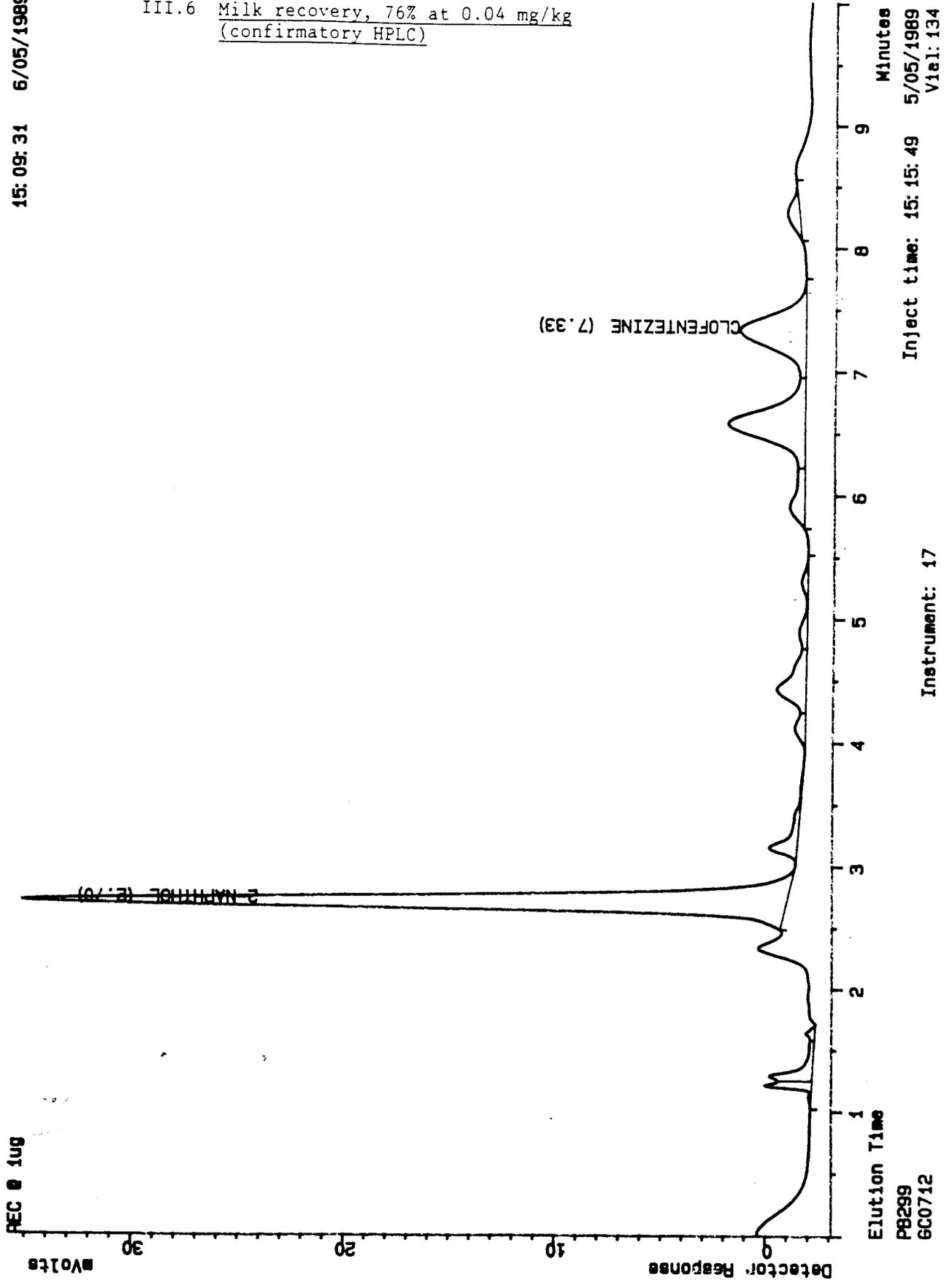
15:08:01 6/05/1989

III.5 Control milk, non-detectable residue
(confirmatory HPLC)

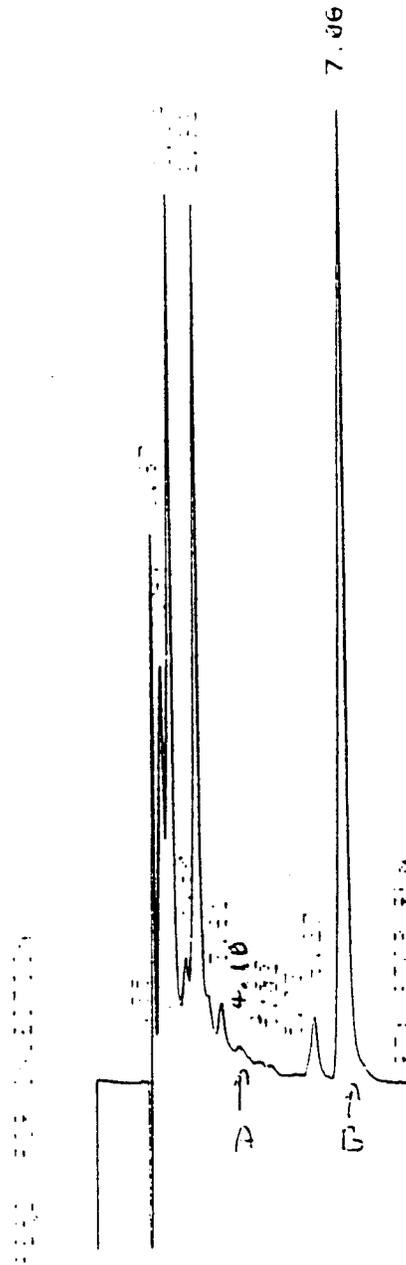


15: 09: 31 6/05/1989

III.6 Milk recovery, 76% at 0.04 mg/kg
(confirmatory HPLC)



III.7 Control fat, apparent residue 0.003 mg/kg



0.003 mg/kg CONTROL FAT INJECTION # 12:32 FEB 3, 1984

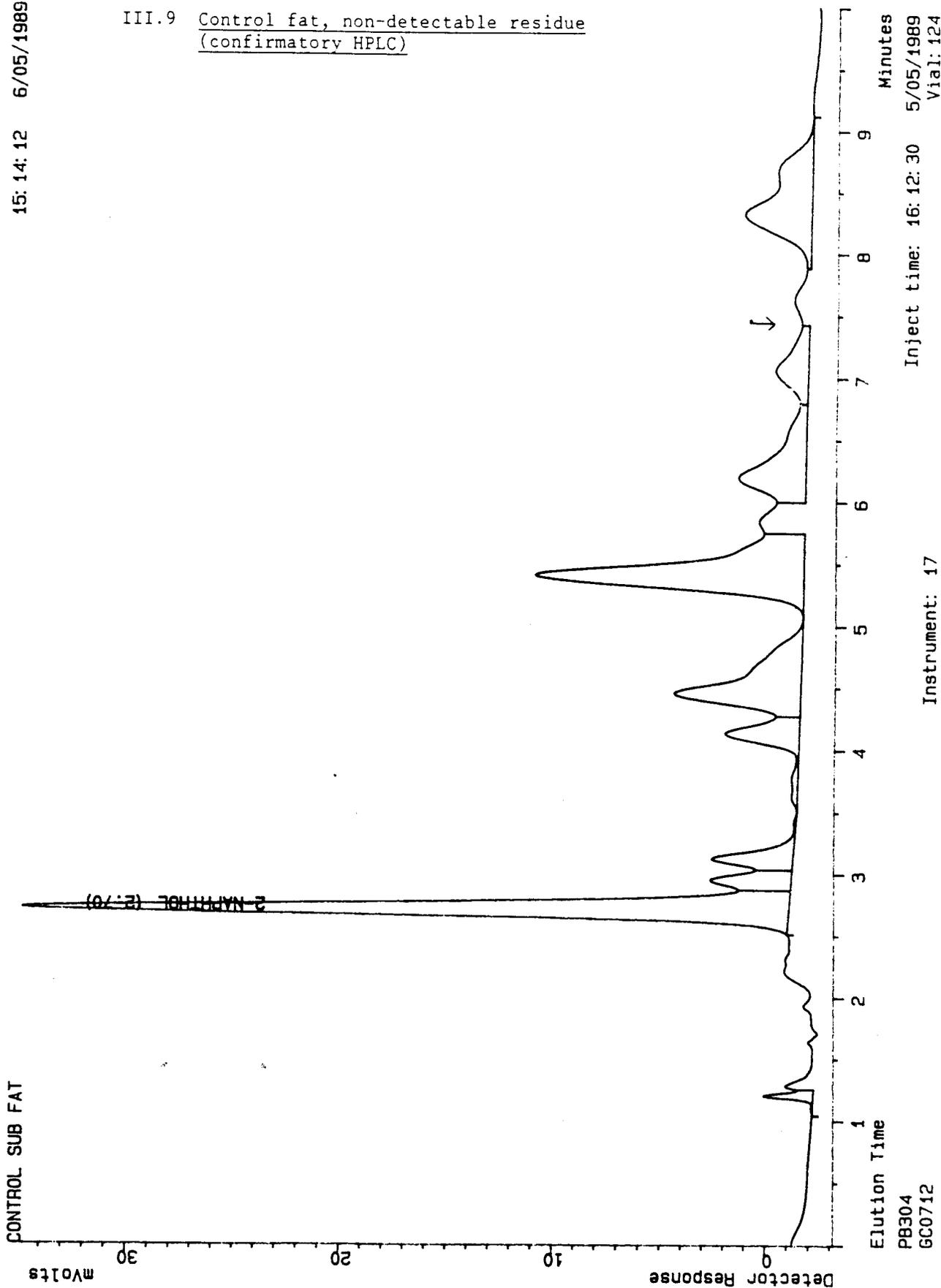
INJECTION # 13804P (1)

RT	HEIGHT	TYPE	RT	NAME
4.10	40.10	SS	0.1277	NO. 1313H
7.06	3550.49	SS	1.5710	018H1710

RATIO = 1.129

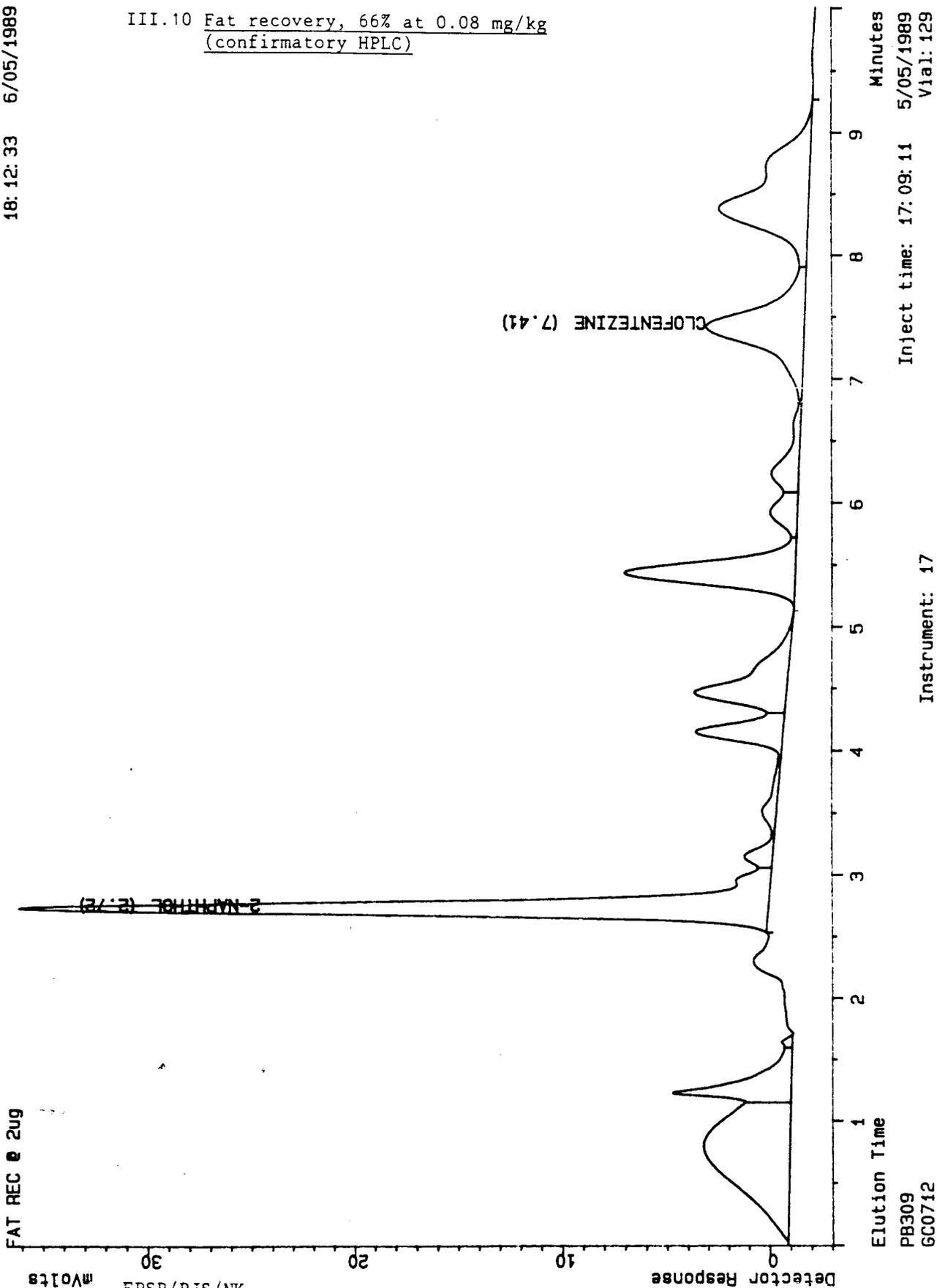
15: 14: 12 6/05/1989

III.9 Control fat, non-detectable residue
(confirmatory HPLC)



18:12:33 6/05/1989

III.10 Fat recovery, 66% at 0.08 mg/kg
(confirmatory HPLC)



FAT REC @ 2ug

mVolts

EPSR/PJS/NK
I.D. 89.50
6 May 1989

Elution Time
PB309
GC0712
Instrument: 17
Inject time: 17:09:11 5/05/1989
Vial: 129
Minutes